

COLLAGEN IN FOSSIL BONE*

BY RALPH W. G. WYCKOFF, ESTELLE WAGNER, PHILIP MATTER, III,
AND ALEXANDER R. DOBERENZ

DEPARTMENT OF PHYSICS, UNIVERSITY OF ARIZONA, TUCSON

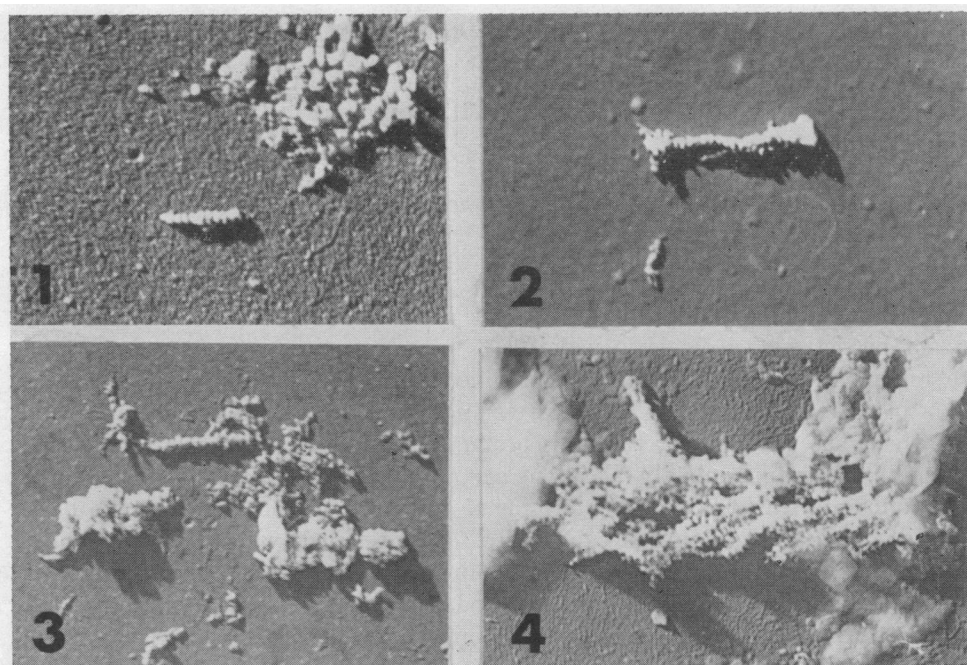
Communicated June 24, 1963

Amino acids have frequently been demonstrated¹ in acid solutions of well-preserved fossils, some of great age. They are undoubtedly often fragments of proteins that originally were present but whose inherent instability is such that they do not persist over geologically significant periods of time. Nevertheless, there are reports of collagen found in mummified human remains² several thousand years old, and in mastodon bones³ preserved under permafrost conditions; and recently it has been stated⁴ that this protein has been identified in the fossilized antlers of a deer-like animal of Miocene age ($\pm 30,000,000$ years). Evidently it is of fundamental importance to determine if any protein, considered as the basic material of all living organisms, can endure, for its detailed examination and analysis could make substantial contributions to our knowledge of evolutionary processes.

The search for such ancient proteins is one aspect of the broader study that can now be made of the microstructure and composition of the fossilized remains of earlier forms of life. Microradiography using X-rays of appropriate wavelengths can establish at the microscopic level whether original structures have been preserved or have been replaced through mineralization; X-ray spectroscopy and diffraction can determine the nature of whatever replacement has occurred. In the case of fossilized teeth, bones, and shells, the calcium phosphate and carbonate that were their original minerals can be selectively removed by chemical means, and microanalyses made of both the organic and inorganic components of the residues thus obtained.

The present paper is a preliminary account of the nature of carbonaceous residues from Early Pleistocene bones collected from the Mendivil Ranch locality (Benson fauna) in Southern Arizona. The specimen analyzed in detail was the heel bone (calcaneum) from an animal of the genus *Equus*⁵.

All samples chosen for study were ones whose fragments were positive for carbon when charred in the absence of oxygen, and whose residues showed no optical or electron microscopic evidence of recent microorganismal invasion. This examination for invasion has been found essential because experience shows that in a fossil the presence of actively growing molds may not be macroscopically apparent. Obviously, subsequent analysis for protein or amino acids is meaningless if such organisms are present. Thick sections of cleaned bone were decalcified by prolonged treatment at room temperature in 1–2.5% trichloroacetic acid, sometimes in the presence of 4% formaldehyde to prevent attack by airborne bacteria. For electron microscopy the residues from this acid treatment, with pulverization either before or after decalcification, were washed repeatedly by centrifugation. A final suspension in water was dried down on the usual collodion-covered grids, shadowed with chromium, and strengthened for microscopy by the vertical evaporation of a thin layer of carbon. Examination was carried out with a small Philips EM75 electron microscope. Fields rich in fibrous material were common, and many of these



FIGS. 1-4.—Electron micrographs of collagen-like fibers recovered from Benson fossil bone. Magnification 16,500 \times .

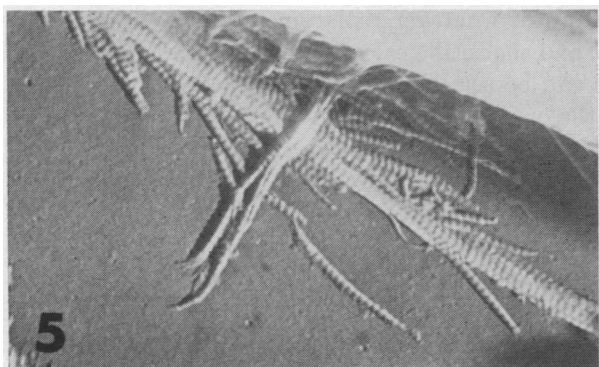


FIG. 5.—Electron micrographs of collagen recovered from desert-dried recent bone. Magnification 16,500 \times .

fibers have a type of regularly striated fine structure (Figs. 1-4) strongly suggestive of collagen (Fig. 5).

Portions of the residues shown by preliminary analysis to contain about two mg of nitrogen were hydrolyzed for chromatography by heating at *ca.* 100°C for 24 hr in 6 *N* HCl followed by evaporation to dryness over hot water. A solution in H₂O of this residue was

poured onto a Dowex 50W-X2 column, and the nonadsorbed cations removed by repeated washings with water. Adsorbed amino acids and peptides, if present, were then eluted by the cautious addition of dilute NH₄OH. The eluate was dried and freed of ammonia by evaporation over hot water; its amino acid content was then determined by unidimensional paper chromatography using phenol as partitioning and ninhydrin as coloring agents. Several controls were run. One type consisted of fossil bones⁶ from the tar pits of La Brea; they were chosen because they are of Pleistocene age, though much younger than the Benson bones, and because the condition of their preservation should be better. After extraction with petroleum ether and the removal of impregnating sand, samples

of the La Brea bone were decalcified in dilute HCl and the residues hydrolyzed as outlined above. Other controls were fresh rat tail tendon, a fresh human tooth, a recent sea lion tooth, and desert-dried calf bone. All these controls, including the La Brea residue, yielded excellent chromatograms which were qualitatively alike and showed the presence of at least the following amino acids: glycine, hydroxyproline, proline, alanine, leucine, glutamic acid, and aspartic acid.

Benson hydrolysates prepared from several samples invariably resulted in definite but weak chromatograms suggestive of about four of the above amino acids. Since their spots cannot be due to free amino acids which would have been eliminated in the original acid solution of the bone, two possibilities exist. They could be derived from an incomplete, insoluble (polypeptide) residue of the initial collagen, or there could be so little collagen remaining in the fossil that a larger sample was needed to provide a complete chromatogram. This second alternative proved correct for, starting with eight gm of fossil bone and employing a more concentrated eluate, a chromatogram was obtained which was qualitatively like those yielded by the controls. It would appear that considerably less than one per cent of the initial collagen remained in the Benson bones. This amount of collagen seemed too small to account satisfactorily for the numerous striated fibers present in the electron microscopic preparations. As Figures 1-4 indicate, the fossil fibers resemble collagen in general appearance and periodicity, but there are significant differences. To facilitate comparison, electron micrographs were made of decalcified fragments of the desert-dried bone. The collagen they contain (Fig. 5) appears identical with fresh collagen. The fossil fibers of Figures 1-3 are markedly shrunk, except longitudinally, compared to those of Figure 5; Figure 4 is typical of large masses of fibers in which the bands are still more reduced in thickness. The repetition of banding along the fossil fibers is *ca.* 600 Å compared with the normal 640 Å collagen spacing. In the electron micrographs of our fossil material, all sorts of transitions between these shrunk fibers are visible, but Figure 1 was chosen to show as close an approach to fresh collagen as has been observed in the Benson fossils. It is hard to avoid the conclusion that the fossil fibers are residual collagen altered with the passage of time.

This raises the important question of whether the change is merely a shrinkage due to a gradual disappearance of much of the initial protein or involves a substitution of inorganic matter. In a preliminary attempt to decide between these alternatives, the residues were examined by X-ray spectroscopy and diffraction. Powder photographs⁷ showed only some clay and a pattern probably attributable to an oxide of uranium, which the spectroscopy demonstrated to have been highly concentrated from the trace detectable in the original bone. The X-ray spectroscopy revealed, besides this uranium, only some iron and calcium possibly associated with the clay. Additional work will be required to show their relation, if any, to the remaining organic matter.

When fresh bone is demineralized by the kind of treatment employed here, there remains a jelly which, though rich in collagen, cannot easily be shredded to free its individual fibers for electron microscopic identification; these are more readily seen in embedded and thinly sectioned preparations. Unlike the Benson material, the La Brea residues behave in similar fashion and, though collagen is demonstrable under the electron microscope, sectioned preparations are needed with them also.

Thus, the present preliminary observations demonstrate that the La Brea collagen is less altered than that in the Benson fossils, but they do not indicate if this should be attributed to their younger age or to the better conditions of preservation that undoubtedly prevailed.

These studies, as extended to bones and teeth of various ages, will be described elsewhere in greater detail.

* This work has been supported by grants from the National Science Foundation (grant 9760) and the National Institutes of Health (grant DE-01183).

¹ Abelson, Philip H., "Organic constituents of fossils," *Geol. Soc. Am. Mem.*, **67**, Part 2, 87 (1957).

² Baud, C. A., and P. W., Morgenthaler, "Recherches sur l'ultrastructure de l'os humain fossil," *Arch. suisses d'anthropologie générale*, **17**, 52 (1952).

³ Randall, J. T., *Nature and Structure of Collagen* (New York: Academic Press, 1953), p. 5.

⁴ Isaacs, W. A., K. Little, J. D. Currey, and L. B. H. Tarlo, "Collagen and a cellulose-like substance in fossil dentine and bone," *Nature*, **197**, 192 (1963); Little, K., M. Kelly, and A. Courts, "Studies on bone matrix in normal and osteoporotic bone," *J. Bone and Joint Surg.*, **44B**, 503 (1962).

⁵ We are indebted to Drs. John F. Lance and Paul A. Wood of the Geology Department, University of Arizona, for these fossils and their identification.

⁶ We wish to thank Drs. Theodore Downs and Reid McDonald of the Los Angeles County Museum for the La Brea fossils.

⁷ The diffraction photographs were prepared by Victor J. Hoffmann.

ON THE STRUCTURE OF COMPACT COMPLEX ANALYTIC SURFACES

BY K. KODAIRA

DEPARTMENT OF MATHEMATICS, JOHNS HOPKINS UNIVERSITY

Communicated by D. C. Spencer, June 10, 1963

The purpose of this note is to outline our recent results on the structure of compact complex analytic surfaces. Details will be published elsewhere. Our proof of the results is based on the Riemann-Roch theorem of which the complete form has been established recently by M. F. Atiyah and I. M. Singer.¹

1. We denote by \mathbf{Z} the ring of rational integers and by \mathbf{C} the field of complex numbers. By a *surface* we shall mean a compact complex analytic surface free from singularities. We consider a surface S and denote by b_ν the ν -th Betti number of S and by c_ν the ν -th Chern class of S . In view of the isomorphism $H^4(S, \mathbf{Z}) \cong \mathbf{Z}$, we may consider c_1^2 and c_2 as rational integers. Letting $\{\Gamma_1, \dots, \Gamma_i, \dots, \Gamma_{b_2}\}$ denote a Betti base of 2-cycles on S , we define b^+ and b^- to be, respectively, the number of positive and negative eigenvalues of the symmetric matrix $(\Gamma_i \Gamma_k)$, where the symbol $(\Gamma_i \Gamma_k)$ denotes the intersection multiplicity of Γ_i and Γ_k . Then we have²

$$b^+ - b^- = -\frac{2}{3}c_2 + \frac{1}{3}c_1^2, \quad b^+ + b^- = b_2. \quad (1)$$

We denote by \mathcal{O} and by Ω^r , respectively, the sheaves over S of germs of holomorphic functions and of holomorphic r -forms. We define

$$h^r = \dim H^r(S, \Omega^r), \quad \Omega^0 = \mathcal{O}.$$